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A Method of Ultraviolet Spectrophotometry of Lipid Monolayers on Silica Gel

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ABSTRACT

A method has been developed for visible and UV spectrophotometry of lipid monolayers after preadsorption on silica gel from solution in hydrocarbon solvents. Lipid-coated silica gel is made optically transparent in the desired spectral region by slurring with an inert solvent mixture. Reproducible gels with nearly the same refractive index as the solvent are achieved by careful choice of mixed solvents and carefully timed settling periods. The gel is pipetted into a silica cuvette of 1 cm light path, and a 9 mm silica spacer bar is inserted between Teflon guide strips. Appropriate choice of solvent mixtures (mainly spectral grade cyclohexane and cyclooctane) permits quantitation of spectra down to 2250 Angstrom units, using a silica gel reference cell. Lipid elution from the monolayer into the solvent is usually less than 1% at the dry-mixing dilutions used. Spectra of known unsaturated carbonyl compounds adsorbed on silica show red shifts as great as 240 Angstrom units, while blue shifts as great as 100 Angstrom units are observed for tocopherol derivatives with an intact aromatic ring. Very small shifts are observed for polyenes. The extent and direction of the shifts are indicative of adsorption and its orientation and are useful in the preliminary determination of the class of an unknown compound. The silica slurry spectra of the important tocopherol oxidation products, the quinone, dimer and trimer, have been examined. Peak wavelength shifts are consistent with structures elsewhere proposed for these compounds.

INTRODUCTION

To study the autoxidation of monolayers of linoleic acid adsorbed from solution at equilibrium onto silica gels of high specific area, it was necessary to develop a method of studying the products while still in the monolayer. This would reduce the risk of confusion caused by artifact formation during elution from the

monolayer. Also, intermediate products, whose survival depends upon steric or mobility restrictions prevailing in the monolayer, could be detected.

Because of the high sensitivity of UV spectrophotometry and the fact that silica gel transmits UV light down to 2200Å, the products were studied by UV spectrophotometry using a gel of the lipid-coated silica in a solvent of approximately the same refractive index as the silica at the wavelength region desired. Scattering effects are minimized by this procedure, although they cannot be eliminated because of the differing refractive indices and dispersions of the lipids, their oxidized products and the silica.

Leermakers and coworkers (1-4) have made the most recent studies on the spectra and photochemistry of adsorbed organic molecules using silica gel in a quartz cell of path length 0.1 cm, added with stirring to a solution of the compound to be studied at appropriate concentration in cyclohexane. Robin and Trueblood (5) studied the spectra of organic molecules adsorbed on silicic acid in cyclohexane slurries previously prepared with magnetic stirring and poured as an aliquot into a quartz cell with path length 0.3 cm. Because of the close proximity of acidic protons of the silanol and hydrogen-bonding field seems to exist at the surface. Thus, pronounced spectral changes both in peak wavelengths and in molar absorptivity may result from adsorption. Leermakers states, "In general, red shifts occur on adsorption of a compound onto the polar adsorbent silica gel if the excited state of the molecule has an increased permanent dipole or if it is more polarizable than the ground state; the blue shifts occur if the reverse is true" (1). The extent and direction of the shifts indicate the fact of adsorption, the nature of the adsorbing group in the molecule and, to a degree, the orientation of adsorption.

EXPERIMENTAL PROCEDURES

Materials

Purifications of Silica Gel G, tocopherol and petroleum ether used in adsorbing monolayers of lipid and reference compounds have been

described elsewhere (6). Neutral alumina (Brockman Activity 1) was purchased from Fisher Scientific Co.

Solvents for silica slurry spectrophotometry were appropriate mixtures of cyclohexane and cyclooctane. The cyclohexane was of spectral grade, purchased from Matheson, Coleman and Bell. Cyclooctane was purchased from Eastman Organic Chemicals, Rochester, N.Y., and Colombian Carbon Company, Princeton, N.J. The cyclooctane was not of spectral grade and contained an impurity adsorbing between 2600 and 2700 Å. This impurity was reduced to approximately one fifth by shaking three times with one half the volume of concentrated sulfuric acid, followed by washes with water and saturated sodium bicarbonate until the effluent was neutral. The product was then dried over magnesium sulfate. Thus purified, it was suitable for silica gel spectrophotometry down to 2100 Å.

Crotonaldehyde was procured from Eastman Organic Chemicals (Rochester, N.Y.). 2,4-Hexadienal and 2,4-hexadienol were procured from Aldrich Chemical Co., Milwaukee, Wisc. Mesityl oxide was procured from Matheson, Coleman and Bell. Eleostearic acid and ethylidene acetone were procured from Pfaltz and Bauer, Inc., Flushing, N.Y. The *d*-alpha tocopheryl quinone was procured from Distillation Products Industries, Rochester, N.Y.

The volatile materials were purified by redistillation. Tocopheryl quinone was purified by column chromatography on silica and gave a single spot in thin layer chromatography. UV absorption values of both tocopheryl quinone and eleostearic acid agreed with those found in literature.

Preparation of the spiro-keto ether dimer and of the allyl and methyl ethers of tocopherol is described elsewhere (7). The trimer of tocopherol was prepared by standard procedures (8), except that final purification was by two repetitions of column chromatography on neutral alumina, using petroleum ether-diethyl ether mixtures, the final elution being with petroleum ether-diethyl ether (19:1) which separates trimer A from trimer B. The characteristics of trimer A, including elemental analysis, were in agreement with values found in literature, with the exception that the IR spectrum (CCl₄) showed a strong 5.93 μ peak, as reported, but neither trimer A nor B showed a 5.80 μ peak.

The method of adsorption of lipids and reference compounds on activated Silica Gel G from petroleum ether solution is described elsewhere (6).

Silica Slurry UV Spectrophotometry

UV spectrophotometry of the lipid-coated silica was conducted in standard silica cuvettes of 1 cm path length and of approximately 4 ml capacity. A reproducible and optically homogeneous slurry of the coated silica was prepared in cyclooctane and cyclohexane solvent mixtures, placed in the bottom of the cuvette, and extruded with a 9 mm quartz spacer bar inserted rapidly into the gel to produce two bubble free, homogeneous layers of slurry totaling 1 mm in path length. Evaporation of the volatile solvent mixture was prevented by a cap of "Saran Wrap" secured with a small rubber band placed over the spacer bar and cell. A reference sample was prepared using uncoated, acid-washed, heat-activated silica in a solvent slurry in a similar cell and spacer combination.

To prepare the silica for UV spectrophotometry a weighed aliquot (about 100 mg) of the coated silica containing autoxidized lipid, which was kept under dry nitrogen at 0°C, was carefully and quickly weighed after restoration to room temperature under dry nitrogen. At the same time, sufficient acid-washed, heat-activated, uncoated silica (about 900 mg) was mixed with the coated silica to compose 1 g. The coated and uncoated silicas were dry-mixed for 5 min in a 50 ml round bottom flask with a small magnetic stirrer bar by manual shaking under dry nitrogen flush at slightly above atmospheric pressure. An escape manifold provided a visual monitor of silica loss, which was kept to a minimum consistent with a steady nitrogen flow.

To form the slurry, exactly 3 ml of a suitable solvent mixture (typically cyclooctane-cyclohexane, 2:8 v/v) was pipetted directly into the bottom of a 25 ml graduated cylinder (19.5 x 1.5 cm) with standard taper orifice and glass stopper, taking care not to wet the walls. A 12 mm magnetic stirrer bar was added and a long stem filter funnel was inserted into the graduate with the stem of a length sufficient to nearly touch the liquid. The mixed silicas or, alternatively, the blank silica, were slowly added with stirring by funnel so that each increment was wetted and incorporated into the slurry without forming a dry powder on the walls of the cylinder. The slurry formed in this manner was almost immediately optically homogeneous; there were no visible bubbles and very little slurry on the walls of the cylinder. At this time, when no clear supernatant was visible, stirring was stopped, the cylinder was stoppered, and the slurry was allowed to settle for a 3 min interval which was carefully timed by a laboratory timer with an

TABLE I

Spectral Shifts of Reference Compounds on Silica

Compound	Wavelength of maximum absorption ^a A units		Spectral shift, cm ⁻¹
	Cyclohexane-cyclooctane solution (8:2 v/v)	Silica gel-cyclohexane- cyclooctane (8:2 v/v) slurry	
Crotonaldehyde	Below 2200	2270	--- (red)
Ethylidene acetone	Below 2200	2295	--- (red)
Mesityl oxide	2315	2470	2711 (red)
Sorbaldehyde	2630	2870	3180 (red)
D-alpha-tocopheryl quinone	2610-2695	2680-2755	808 (red)
Sorbyl alcohol	2295	2295	0 (none)
Beta-eleostearic acid	2705	2720	204 (red)
D-alpha-tocopherol	2980	2880	1165 (blue)
Tocopheryl allyl ether	2880	2850	365 (blue)
Tocopheryl methyl ether	2880	2850	365 (blue)
Keto ether dimer of tocopherol	3020	2950	786 (blue)
	3410	3770	2800 (red)
Trimer of tocopherol	2945	2910	408 (blue)
	---	2525	--- (red)

^aBased on slant baseline. Method described in text.

alarm. The reference slurry was mixed first; after it had settled approximately 1½ min, the 3 min settling period for the coated sample was begun and timed in the same manner.

At the end of the timed period, the volume of the slurry was 3.25 ml. An 0.8 ml aliquot of the slurry was slowly and carefully withdrawn from a position 2 mm above the bottom of the graduate using a finger-controlled pipette filler and a pipette prepared from a fire-polished glass tube 4 mm i.d., scratch-marked at a suitable point. Seven tenths of a milliliter of the slurry sample was deposited in the open cuvette at a point 2 mm from the bottom and the 9 mm quartz spacer bar was thrust quickly and firmly into the slurry, extruding it up the cell walls. To prevent the development of cracks in the slurry, it was found that this operation must be performed quickly and preferably by two people. The Saran Wrap cell cover was secured, the cell surfaces gently wiped, and the cell placed in the spectrophotometer cuvette holder. The effective life of such a preparation is approximately 10 min before cracks and bubbles develop. Therefore, a calibration baseline was made using the cuvettes alone or filled with liquid solvent instead of slurry. Experience had proven the adequacy of such a procedure for precision; the rapidity of onset of deterioration of the reference slurry prevented its use

against both a blank and the sample. The deterioration is relatively sudden and immediately detectable by eye. Prior to this, repetitions of optical absorbance never differ more than 5%, and changes in the position of the peak wavelengths are not observable.

Spectra were read on a Cary Recording Spectrophotometer, Model 14M, using a slit control setting of 50 and full slit height. Under the above conditions, spectra could be obtained between 2250 and 3600 Å using the 2/8 cyclooctane-cyclohexane solvent mixture, the shorter wavelength limit being set by the absorbance of the reference silica and the consequent increase in slit width. In general, an initial preparation was followed by a repeat preparation of both sample and reference slurries. Optical repetitions on the same slurry differed so little that they were not included in the standard procedure. Preparative repetitions from the same dry mixture of silica in general differ less than 5% in absorbance, and even when preparations are made from separately weighed and mixed silica samples, the cumulative variability in molar absorptivity is less than 10%. The wavelengths of maximum absorption are very reproducible, particularly above 2400 Å, but errors inherent in slurry preparation and settling permit less precision for molar absorptivities.

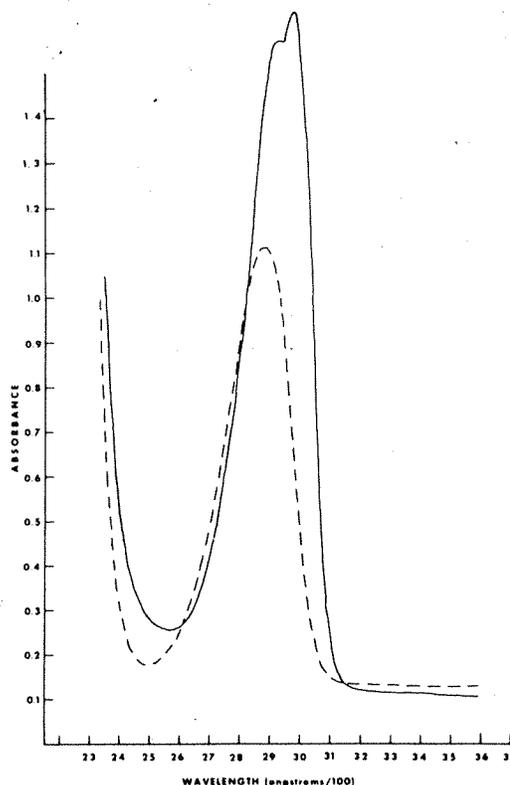


FIG. 1. UV spectrum of d- α -tocopherol. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.176 mg/ml.

RESULTS AND DISCUSSION

Selection of Slurrying Solvent

Very few solvents are suitable for performing UV spectrophotometry with silica precoated with lipid monolayers. They cannot have any important UV absorption above 2100 Å. They must be relatively inert chemically, and, preferably, aliphatic hydrocarbons. More polar materials will strongly elute products from the silica surface. Solvents must be relatively nonvolatile and possess an index of refraction high enough to match the lipid-coated silica in the ranges desired. The components of a solvent pair must be suitably different in index so that matching mixtures may be found for the desired range. It is also important that the supernatant be clear, to insure that all the coated silica particles are being included in the measured volume after settling of the slurry. More polar solvents fail to produce such coherence of the slurry. The cyclooctane-cyclohexane combination in various proportions was found to be the best.

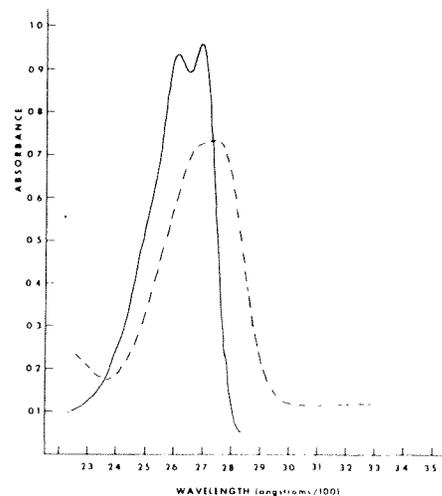


FIG. 2. UV spectrum of d- α -tocopheryl quinone. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.023 mg/ml.

Optical Limitations of the Method

To increase transparency of oxidized lipid-coated silica slurries in the 2200 to 2500 Å regions where diene hydroperoxide and alpha-beta unsaturated carbonyl compounds absorb, cyclooctane-cyclohexane mixtures were used, rather than cyclohexane alone (Robin and Trueblood, and Leermakers). However, any spectrophotometric studies of silica solvent slurries are limited by the fact that the refractive dispersions (increase of refractive index with decreasing wavelength) of liquids are generally greater than those of solids. A good match at one wavelength is an imperfect match at another wavelength, i.e., a slurry nearly transparent in the UV may be only translucent in the visible range. This is responsible for the well known Christiansen-filter effect of enhanced transmission of a slurry in the range of best match of solvent and solid indices of refraction (5). To this is added the effect of the small particle size of the silica, which always tends to increase Rayleigh scattering toward lower wavelengths. Furthermore, the lipid which is in the monolayer coating on the silica usually has a different index of refraction from either the silica or the solvent, and this difference is strongly enhanced upon oxidation. Thus a perfect match of solvent and coated solid, even at one wavelength, is impossible, and a compromise is necessary in choosing solvent mixtures for the particular lipid material and wavelength region of study. In addition, a solvent refractive index which matches the refractive index of the uncoated reference

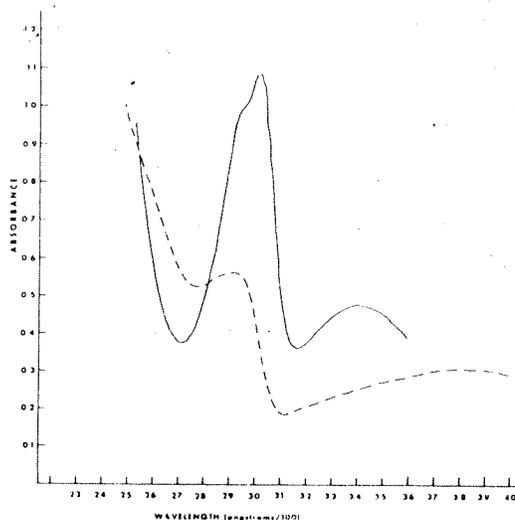


FIG. 3. UV spectrum of keto-ether dimer of d -alpha-tocopherol. Cyclooctane-cyclohexane, 2:8 (solid line, 0.192 mg/ml). Cyclooctane-cyclohexane-silica slurry (dashed line, 0.128 mg/ml).

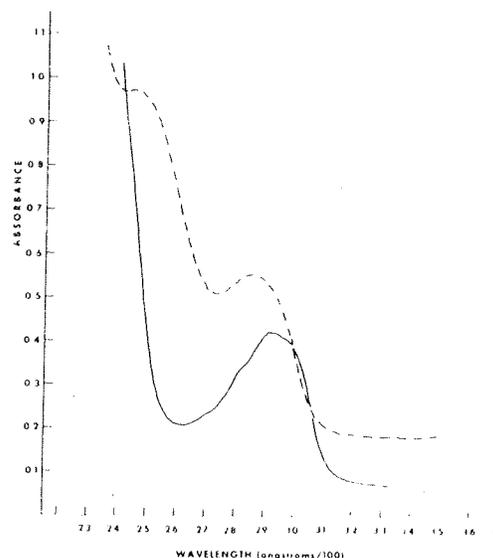


FIG. 4. UV spectrum of tocopherol trimer A. Cyclooctane-cyclohexane, 2:8 (solid line); cyclooctane-cyclohexane-silica slurry (dashed line); 0.086 mg/ml.

silica, and hence extends the spectral range to lower wavelengths, may add to the scattering background of the coated silica sample which appears like an "end-absorption" or a slowly rising background toward shorter wavelengths.

The extinction of uncoated silica slurries, prepared as above in cyclooctane-cyclohexane (2:8), showed the following values at representative wavelengths: wavelength 2300 A, 1.5 extinction; 2900 A, 0.4; 3600 A, 0.5.

Theoretical maximum transmission or the Christiansen "window" (3) should occur nearer to 2300 A, but the nonselective increase of Rayleigh scattering with decreasing wavelength shifts this point toward the red. In general, however, the optical density of these slurries is low, compared to the dense, light-scattering materials for which Butler (9) reports selective wavelength-dependent scattering due to anomalous dispersion in the region of absorption bands. Anomalies due to stray light and fluorescence found in dense, light-scattering materials are also minimized by the much lower optical density of correctly matched solvent-silica slurries.

Peak Wavelength and Absorbance Measurements

In practice the effects of background scattering can be minimized if peak wavelengths are located and absorbances are measured above a slant base line. The points of tangency with the apparent upper and lower wavelength limits of the peak were connected by a straight line. Peak wavelength was the wavelength of the

point of tangency of the tangent to the curve which was parallel to this slant base line. Peak wavelengths, so defined, agreed well with those of the identical compounds in the absence of background scattering, whereas the apparent peak positions (above an assumed horizontal base line) are the sums of the background and compound absorbances, which always shift the apparent location of a smaller peak toward that of the larger peak upon which it is superimposed. Absorbance was determined as the area bounded by the curve above the slant base line, which was found to be directly proportional to the scalar absorbance measured between the slant base line and the determined peak parallel to the absorbance axis of the chart paper.

Extent of Elution by Slurrying Solvent

To test the extent of elution of relatively nonpolar compounds by the slurrying solvent during the course of the silica-UV spectrophotometry, the supernatant from a slurry coated with a monolayer containing 5 moles per cent tocopherol in stearic acid was examined. The slurry was prepared without any dry-mixing dilution of the coated silica. The supernatant showed less than 8% of the total tocopherol present in the system. Under the condition of 1:10 dry-mixing dilution, the supernatant contamination by elution is reduced to vanishingly low levels. Since the usual powder dilution is 1:10, the method gives reasonably quantitative absorbance values and

highly reproducible wavelengths of maximum absorption referable to compounds adsorbed in the monolayer rather than dissolved in the solution phase.

Silica-UV Spectrophotometry of Reference Compounds

To identify the absorption bands present in the silica-UV spectrum resulting from a mixture of reaction products, the wavelengths of maximum absorption and the molar absorptivity of authentic analogous reference compounds were determined on silica. This procedure permits tentative resolution of a mixed spectrum into its components. It also aids greatly in tentative classification of unknown compounds, since the extent of red and blue spectral shifts are very diagnostic. Large shifts also confirm that the moiety of a compound producing the shifted band is within the silanol electrostatic field, if not directly hydrogen-bonded, and thus confirm location and orientation in the monolayer.

Table I gives a list of relevant purified reference compounds whose spectral indices have been determined in solvent and on silica in this laboratory.

As discussed above in connection with tocopherol, red shifts appear characteristic of the $\pi-\pi^*$ transition of quinones, unsaturated aldehydes and ketones; blue shifts appear to be characteristic of the similar transition in aromatic compounds with electron-donating substituents. Little or no shift occurs for polyenes without a conjugated donating or withdrawing group.

Our work has indicated that tocopherol and its derivatives with intact aromatic and chroman ring structure experience a blue shift in maximum and a decrease in molar absorptivity from the solvent values upon adsorption on silica (Fig. 1), whereas dienes or monoenes conjugated with a carbonyl group as in tocopheryl quinone (Fig. 2) or the dienone group of the dimer (Fig. 3) experience a red shift.

Thus, the tocopherol dimer, whose proposed structure (10,11) has separate aromatic and homoannular diene ketone moieties, not conjugated with each other, would be expected to have a moderate blue shift of the 3020 Å band and a very strong red shift of the 3410 Å band. A decrease in absorbance of the 3020 Å band would be also expected. These changes were found (Fig. 3), giving further confirmation to the proposed structure, about which doubts have been recently expressed (12). The tocopherol trimer (8) is reported to contain two aromatic rings with associated chroman groups, together with an alpha-beta unsaturated cyclic

ketone. The silica slurry spectrum (Fig. 4) showed the expected blue shift of the aromatic band at 2945 Å and the red shift of the shorter wavelength absorption of the unsaturated carbonyl (appearing as end absorption below 2400 Å in the solvent spectrum).

The small blue shift of the tocopheryl ethers in contrast to that of tocopherol and the chroman group of the dimer, appears to result from the anomalously short wavelength of their $\pi-\pi^*$ band (2880 Å in the solvent cyclohexane-cyclooctane, 8:2). The ethers are examples of molecules with the steric inhibition of resonance found in 2,6-dialkylated, alkyl-aryl ethers (13). Since donation of lone pair electrons to the ring is thus reduced, the wavelength of maximum absorption in solution is abnormally short and bonding to silica produces less blue shift.

Unstrained six-membered ring formation involving the ether oxygen is known to remove these steric distortions. Therefore, in tocopherol and the aromatic band of its dimer, wavelengths in solvent are longer and blue shifts due to silica bonding are greater. The shorter wavelength of the aromatic band of the trimer in solvent in contrast to that of the dimer, and its smaller blue shift in silica slurry are consistent with greater steric distortion of the chroman rings than that in tocopherol and the dimer. The proposed molecular structure (8) would be expected to show steric strain.

Thus, for a given type of compound to be expected from a surface reaction, authentic analogue compounds may be studied in the adsorbed state on silica to determine the wavelengths of maximum absorption and the molar absorptivity. The amount of a known compound adsorbed may be calculated and the components of a spectrum resulting from two or three compounds present on the silica may be tentatively predicted and confirmed upon elution and separation. In addition, unknown compounds eluted from the silica surface and separated by appropriate methods may be classified by the extent and direction of the wavelength and absorptivity shifts of their spectra in solution as compared to those in silica slurry. This affords a valuable clue to the nature of the compound.

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